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Comparison of the Interferon-Tau Expression from Primary Trophectoderm Outgrowths Derived from IVP, NT, and Parthenogenote Bovine Blastocysts

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ABSTRACT The expression of interferon-tau (IFN-τ) is essential for bovine embryo survival in the uterus. An evaluation of IFN-τ production from somatic cell nuclear transfer (NT)-embryo-derived primary trophectoderm cultures in comparison to trophectoderm cultured from parthenogenote (P) and in vitro matured, fertilized, and cultured (IVP) bovine embryos was performed. In Experiment 1, the success/failure ratio for primary trophectoderm colony formation was similar for IVP and NT blastocysts [IVP = 155/29 (84%); NT 104/25 (81%)], but was decreased ($P = .05$) for P blastocysts [54/43 (56%)]. Most trophectoderm colonies reached diameters of at least 1 cm within 3–4 weeks, and at this time, 72 hr conditioned cell culture medium was measured for IFN-τ concentration by antiviral activity assay. The amount of IFN-τ produced by IVP-outgrowths [4311 IU/mL (n = 155)] was greater ($P < .05$) than that from NT- [626 IU/mL (n = 104)] and P - [1595 IU/mL (n = 54)] derived trophectoderm. Differential expression of IFN-τ was confirmed by immunoblotting. In Experiment 2, colony formation was again similar for IVP and NT blastocysts [IVP = 70/5 (93%); NT 67/1 (99%)] and less ($P < .05$) for P blastocysts [65/27 (70%)]. Analysis of trophectoderm colony size after 23 days in culture showed a similar relationship with P-derived colonies being significantly smaller in comparison to IVP and NT colonies. A differential expression of IFN-τ was also observed again, but this time as measured over time in culture. Maximal IFN-τ production was found at day-14 of primary culture and diminished to a minimum by the 23rd day. *Mol. Reprod. Dev. 75: 299–308, 2008.* Published 2007 Wiley-Liss, Inc.†

Key Words: interferon-tau; bovine; trophectoderm; cell; culture

INTRODUCTION

Somatic cell nuclear transfer (NT) can create animals from cultured somatic cells by using the cultured cell's nucleus to replace the oocyte's nucleus. However, NT embryo development to term and post-natal survival is usually dramatically lower than that of embryos produced naturally or by in vitro maturation, fertilization, and culture (IVP) (Wells et al., 1997; Wilmut et al., 1997; Cibelli et al., 1998; Wakayama et al., 1998; Shu-Hung et al., 2002). Incomplete nuclear reprogramming, that is, the molecular events that return the somatic cell's nuclear material to a state similar to that of the zygote's nuclear material, may be the major cause of the high percentage of developmental failures of NT embryos (Surani, 2001; Humpherys et al., 2001). For example, conservation of appropriate chromatin methylation patterns appears to be a problem in NT embryos (Bourc'his et al., 2001; Kang et al., 2002; Cezar et al., 2003; Santos et al., 2003). Moreover, numerous differences in gene expression patterns have been found in embryos and fetuses produced from normal fertilization with sperm in comparison with those produced by NT (Humpherys et al., 2002; Inoue et al., 2002; Niemann et al., 2002; Suemizu et al., 2003). Various developmental abnormalities apparently arise from these epigenetic NT reprogramming errors, and among the

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most common are deficiencies in placentation or placental hyperplasia (Hill et al., 2000; Tanaka et al., 2001).

Failure to form a normal placenta is apparently responsible for many NT pregnancy losses in cattle (Hill et al., 2000; De Sousa et al., 2001; Hashizume et al., 2002; Lee et al., 2004; Patel et al., 2004). The bovine embryo's trophectoderm tissue, which mediates placental connections with the uterine epithelium of the cow, may be subject to deleterious epigenetic changes resulting from reprogramming errors after NT. The recent report that certain DNA methylation anomalies were specifically localized to the trophectoderm cells of bovine NT blastocysts suggests that the analysis of trophectoderm tissue might be useful in determining epigenetic deficiencies that lead to placental dysfunction (Kang et al., 2002).

One aspect of early NT embryo function that may be influenced by NT reprogramming and that is required for the establishment of pregnancy is the expression of the trophectoderm secretory protein interferon-tau (IFN-τ). Sufficient quantities of IFN-τ must be secreted in order for pregnancy to be established and maintained in cattle and other ruminants (Roberts et al., 1992, 1989; Bazer et al., 1997). Gene expression of IFN-τ begins as the trophectoderm is first forming at the late morula and early blastocyst stage of development, and it is expressed constitutively until the trophectoderm begins to attach to the uterine lining on or after day 19 of pregnancy in cattle (Helmer et al., 1987; Hernandez-Ledezma et al., 1992; Kubisch et al., 1998; Ealy et al., 2001). The primary physiological action of IFN-τ is to limit prostaglandin F2α pulsatility from the endometrium and thereby prevent regression of the corpus luteum so that a pregnant state can be maintained (Bazer et al., 1997; Thatcher, 1997). In addition to this, it is likely that IFN-τ effects the expression of numerous endometrial genes that may determine the success or failure of placentation (Pru et al., 2001; Nagaoka et al., 2003; Martin et al., 2004; Gray et al., 2006; Klein et al., 2006). Evidence from various studies suggests that the expression of IFN-τ during early pregnancy may be affected by nuclear reprogramming events. For example, increases in DNA methylation within the 5′-flanking region of the ovine IFN-τ gene decreases gene expression, and this may account for the repression in IFN-τ expression after placentation (Nojima et al., 2004). Also, Wrenzycki and co-workers demonstrated that the expression of IFN-τ was up-regulated in bovine blastocysts derived from nuclear cloning compared to those derived from IVP or from in vivo fertilization and development (Wrenzycki et al., 2001). Thus, the functional importance IFN-τ and its apparent sensitivity to nuclear reprogramming events indicate that it might be used as a marker of successful nuclear reprogramming in NT bovine embryos.

We have previously shown that the culture of IVP- or parthenogenote-derived bovine trophectoderm tissue on STO feeder cells reproduces some aspects of the intact preimplantation bovine embryo's trophectoderm tissue such as its cell growth, morphology, and expression of IFN-τ (Talbot et al., 2000a,b). This in vitro model was employed in a comparison of IFN-τ secretion levels from primary trophectoderm outgrowths of NT, parthenogenetic (P), or IVP bovine 8–11-day blastocysts.

MATERIALS AND METHODS

Experiments 1 and 2

Experiment 1 consisted of an assessment of IFN-τ secretion from independent primary trophectoderm colonies that had reached a minimum of 1 cm in diameter over time in culture and was designed to compare IFN-τ activity or content by method of blastocysts production, i.e., somatic cell nuclear transfer (NT), in vitro matured, fertilized, and cultured (IVP), or parthenogenesis (P). Specific variables not held constant in Experiment 1 included the following: source of egg, source of sperm, in vitro egg maturation method, age of blastocyst (day-8, -9, -10, or -11 of in vitro egg culture) when primary trophectoderm culture was initiated, the bovine fetal fibroblast cell line used as nuclear donors for NT, length of time of trophectoderm primary culture (within 2.5–4 weeks), and time of STO feeder-layer preparation. Two diameter measurements at right angles to one another were taken of each colony at the time 72 hr conditioned medium (CM) was collected for measurement of IFN-τ activity or content (see below).

Experiment 2 assessed IFN-τ secretion from independent primary trophectoderm colonies by blastocyst production method (NT, IVP, or P) and by days in primary culture (day-14, -17, -20, and -23). Specific aspects of Experiment 1 that varied (see above) were held constant in Experiment 2, i.e., source of egg, source of sperm, in vitro egg maturation method, age of blastocyst (day-8 of in vitro egg culture) when primary trophectoderm culture was initiated, the bovine fetal fibroblast cell line used as nuclear donor for NT, STO feeder-cell source (irradiated/frozen cell stocks) and time of feeder-layer preparation (1 week prior to initiation of primary trophectoderm culture). Seventy-two hour CM collected at day-14, -17, -20, and -23 was assayed for IFN-τ antiviral activity. On day 23 of culture the size of the primary trophectoderm colonies were assessed by taking two colony diameter measurements at right angles to one another.

Statistical Analysis

The assumptions of the general linear model were checked. When necessary to correct for variance heterogeneity the data was log transformed. The means presented in the tables are given in the original units. Whenever means comparisons were made, Sidak adjusted *P*-values were used so that the experiment-wise error was 0.05.

Analysis of Experiment 1

Success or failure of colony-formation was analyzed by blastocyst production method and success/failure as a 3×2 contingency table using StatXact (Cytel Software). An exact Pearson chi-square test was completed to

determine if the distribution of colony-formation was the same for the three independent populations defined by blastocyst production method. Method was statistically significant ($F = 30.61$; $P = .0000$; d.f. $= 2$). Pair-wise comparisons of the three populations were done (Table 1).

Experiment 1 IFN-τ levels (IU/ml) were statistically analyzed by blastocyst production method (IVP, NT, and P). The analysis was performed by partitioning the data into nine complete blocks based on the combination of egg source, days in egg culture, and days in tissue culture. After defining the nine blocks and omitting data with missing or zero values, there remained 219 observations for the analysis. To meet the assumptions of the general linear model, the data were log transformed. The transformed data were analyzed as a two-factor mixed linear model using Proc Mixed (SAS Institute, Cary, NC) with Method as the fixed effect and Block as a random block. Method was statistically significant ($F = 61.95$, $P < .0001$, d.f. $= 2$). Means and mean comparisons are given in Table 2.

Analysis of Experiment 2

Colony-formation data were analyzed the same as for Experiment 1 (Table 1). Method was statistically significant ($F = 30.14$; $P = .0000$; df $= 2$).

Experiment 2 IFN-τ activity data were analyzed as a two-factor repeated measures linear model using Proc Mixed with Method (IVP, NT, and P) and Days in egg culture (14, 17, 20, and 23) as the repeated factor. To meet the homogeneous variance assumption of the general linear model the data were log transformed for the analysis. The unstructured variance–covariance model was found to best account for the correlation in Days in egg culture. The Method × Day interaction was statistically significant (Table 3). Means and mean comparisons are given in Table 4.

Experiment 2 colony size data were analyzed as a one-factor linear model with embryo production method as the factor. Method was statistically significant ($F = 20.96$, $P < .0001$, d.f. $= 2$). Means and mean comparisons are given in Table 5.

In Vitro Production of Bovine Blastocysts

Parthenogenetic, NT, and IVP bovine blastocysts were produced as previously described (Powell et al., 2004). For Experiment 1, embryos were produced from in vitro matured cumulus-oocyte complexes (COC) processed from local slaughterhouse ovaries (Mopac) or obtained from Bomed Inc., Madison, WI. For Experiment 2, in vitro matured COC were obtained from Bomed Inc. Embryo culture was in G1/G2 medium (Vitrolife, Englewood, CO) and an atmosphere of 5% oxygen, 5% CO_2, and 90% nitrogen for both Experiments 1 and 2.

In Experiment 1 for IVP, three bulls were used as a source of sperm. One bull was a high genetic stock Holstein. The second was a chimera derived from the aggregation of three morula, one of which was a transgenic NT morula. This chimeric bull showed germ line contribution from the transgenic NT morula as evidenced by nuclear localized green fluorescent protein (GFP) expression in approximately 40% of resultant blastocysts. The third bull was a transgenic NT Jersey bull and again had nuclear localized GFP as one of three engineered transgenes. For Experiment 2, the same high genetic stock Holstein bull was used to produce IVP embryos as in Experiment 1.

Three bovine fetal fibroblast (BFF) cell lines were used to provide nuclear donor cells for the production of NT embryos in Experiment 1. The origin of the BFF 10 cell line has been previously described (Powell et al., 2004), and this cell line was the nuclear donor in approximately three-quarters of the NT embryos produced in the study. A second cell line (BFF 13) was derived from a 100-gestational day cloned fetus that was cloned from the third cell line which consisted of an adult fibroblast culture (BFF 101) established from a BFF 10-derived transgenic NT cow muscle biopsy. For Experiment 2, the BFF 10 cell line was used for NT nuclear donor cells.

Cell Culture

The following cell culture conditions applied to both Experiments 1 and 2. All cells were grown on tissue culture plastic ware (Nunc, Denmark; Falcon, Becton/Dickinson, Lincoln Park, NJ). Fetal bovine serum (FBS) was obtained from Hyclone, Logan UT. Cell culture reagents including Dulbecco's phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}, media, trypsin–EDTA (0.05% trypsin, 0.43 mM EDTA), antibiotics, non-essential amino acids, and L-glutamine were purchased from InVitrogen Corporation (GIBCO), Gaithersburg, MD. STO cells (CRL 1503, American Type Culture Collection, Rockville, MD) were grown in

TABLE 1. Dependence of Formation of Trophecotoderm Colonies on Method Used to Generate Blastocysts

	Experiment 1		Experiment 2	
	Colonies formed		Colonies formed	
Method	Blastocysts cultured	Percent	Blastocysts cultured	Percent
Fertilized	155/184	84.2 a*	70/75	93.3 a
Nuclear transfer	104/129	80.6 a	67/68	98.5 a
Parthenogenic	54/97	55.7 b	65/92	70.1 b

*Methods with different letters are different at the 0.05 significance level.

TABLE 2. Means and Means Comparisons of Experiment 1: Affect of Blastocyst Production Method on IFN-τ Expression from Trophectoderm Outgrowths

Method	Mean production (IU/mL)
Fertilized	4228.63 a*
Parthenogenic	2419.91 b
Nuclear transfer	422.67 c

The means are transformed back to the original units from the statistical analysis.
*Methods with different letters are different at the .05 significance level.

Dulbecco's modified Eagles medium with high glucose supplemented with 10% FBS (10% DMEM). Feeder layers were prepared by exposing a suspension of STO cells to 8 krad gamma radiation and plating the cells at 6×10^4 cells/cm^2. Feeder layers were maintained by refeeding with 10% DMEM every 6–7 days.

In Experiment 1, primary cultures of trophectoderm from 8- to 11-day blastocysts were individually cultured in 4-well plates with STO feeder layers. The primary cultures were initiated as previously described by pressing the blastocysts onto the STO feeder cell monolayer and plastic surface with 27 G hypodermic needles (Talbot et al., 2000b). For the IVP group, the blastocyst used were hatched or hatching. For the P group, blastocyst were seldom hatching, but all were largely expanded with zona pellucida just visible under the dissection microscope as a thin outer ring. A few of the NT blastocysts were at a stage where they were beginning to protrude through the slit in the zona pellucida made by the enucleation micropipet. However, most of the NT blastocysts used had expanded to the extent that they had formed a typical dumbbell-shaped "hatching" blastocyst with the protrusion of a significant proportion of the blastocyst's cells outside the zona pellucida. Primary trophectoderm cultures were maintained under 1 mL of 10% DMEM with refeeding every 3–4 days. Any contaminating endoderm cells were removed completely by dissection as described previously (Talbot et al., 2000b). Primary trophectoderm colonies were grown for 2.5–4 weeks until they reached 1–1.5 cm in diameter, and 72 hr CM was collect and frozen at −70°C at the end of the culture period.

For Experiment 2, the initiation and culture of primary trophectoderm colonies was as above for Experiment 1 except that all cultures were initiated from 8-day blastocysts. Also, 72 hr CM was collected at day-14, -17, -20, and -23 of culture, and the diameter of the colonies were measured on day 23 (the last day of culture).

TABLE 3. Analysis of Variance

Source	d.f.	*F*-value	*P*-value
Method	2	26.27	<.0001
Day	3	324.67	<.0001
Method × Day	6	8.60	<.0001

TABLE 4. Means and Means Comparisons of Experiment 2: Antiviral Activity of Primary Trophectoderm Colonies by Days in Culture and Blastocyst Production Method

	Method		
Day	IVP	NT	Parth
14	10,887 a*x†	3048 ay	7213 ax
17	3662 bx	912 by	3231 bx
20	1767 cx	511 cy	1133 cx
23	1053 dx	390 dy	588 dy

*Day means within blastocyst production method with different a, b, c, d letters are different at the .05 significance level.
†Method means within Day with different x, y letters are different at the .05 significance level. Means are expressed in original units of IU/mL.

Antiviral Interferon-Tau (IFN-τ) Activity Assay

IFN-τ concentration was determined in CM from each primary trophectoderm culture. Antiviral assays of the CM were completed as described by Roberts et al. (1989). Conditioned medium from STO feeder cells alone and unconditioned medium were assayed as negative controls. The ability of samples to prevent virus-induced cell lysis by 50% was compared to a recombinant human interferon-alphaA standard (Calbiochem, La Jolla, CA; 3.84×10^8 IU/mg). The concentration of IFN-τ in CM was calculated based on the specific activity of recombinant bovine IFN-τ (rbIFN-τ; $2.52 \pm 0.49 \times 10^8$ IU/mg) included in each assay. Assays were completed in duplicate and results were reported in international units (IU) of IFN-τ per mL of culture medium. The assay had a sensitivity of 20 IU/mL.

Immunoblot Analysis of Conditioned Medium (CM)

Electrophoretic separation and Western blotting of the proteins in selected samples of 72 hr CM (1 ×) from primary trophectoderm outgrowths were immunoprobed with anti-bovine IFN-τ antibody as described previously (Talbot et al., 2000b). Proteins were transferred on to PVDF membranes (Millipore, Bedford, MA) and ECL Plus (Amersham Biosciences, Piscataway, NJ) was used for antibody detection. Anti-bIFN-τ antibody, used at a dilution of 1:2500, was the kind gift of Dr. Michael Roberts (Klemann et al., 1990).

RESULTS

Experiment 1

Formation of primary trophectoderm colonies was dependent on the method used to generate blastocysts

TABLE 5. Method Means and Means Comparisons

IVP	1.183a*
NT	1.120a
Parth	0.862b

*Method means with different letters are different at the .05 significance level.

(Fig. 1). Incidence of colony-formation did not differ between IVF-derived and NT-derived blastocysts, but fewer ($P < .05$) P-derived blastocysts were able to form outgrowths (Table 1).

IFN-τ is expressed soon after bovine blastocyst formation and therefore might be a useful marker of competency for embryos produced by NT. To assess this possibility, a comparison was made of IFN-τ levels in the 72 hr CM of primary trophectoderm cultures derived from IVP, NT, or parthenogenic (P) bovine 8–11-day blastocysts. Expression of IFN-τ from trophectoderm outgrowths was affected by the method used to generate blastocysts. Mean antiviral activity of CM was greatest in IVF-derived outgrowths, intermediate in P-derived outgrowths, and lowest in NT-derived outgrowths (Table 2).

IFN-τ levels were measured in selected samples by probing Western blots of CM with anti-bovine IFN-τ antibody, and these values were compared with the corresponding antiviral activity of the samples. The immunoblot analysis verified that IFN-τ was produced by the primary trophectoderm cultures (Fig. 2). No IFN-τ was detected in the CM of STO feeder cells alone or in unconditioned cell culture medium (Fig. 2, lanes 11 and 12, respectively). Densitometry measurements taken of the 20–23 kDa differentially glycosylated IFN-τ bands of the immunoblots correlated well with antiviral titers (Fig. 3). The log transformation plot of antiviral activity versus immunoblot signal density showed a straight line relationship with a correlation coefficient of 0.91 ($P = .001$) (Fig. 3).

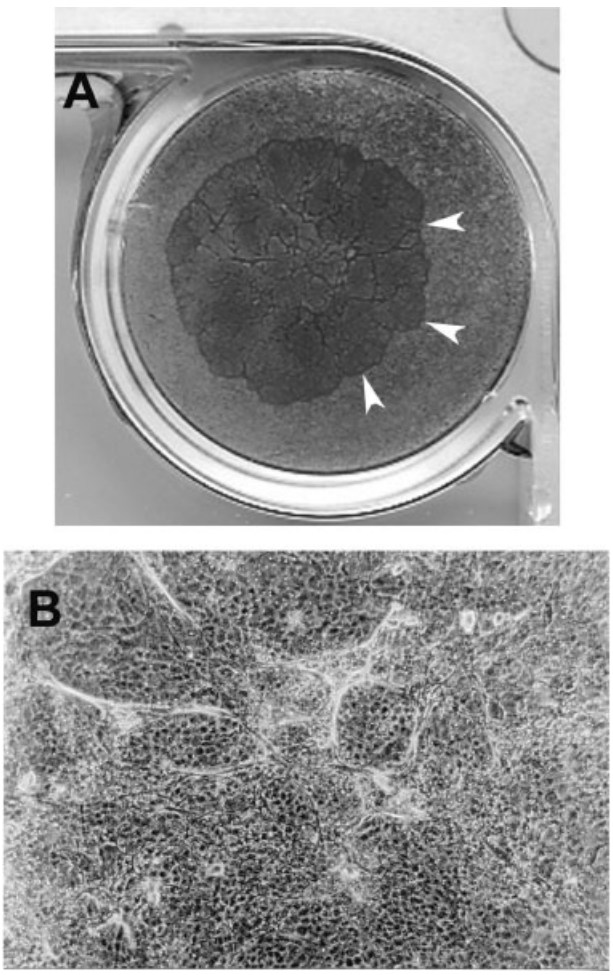

Fig. 1. Primary bovine trophectoderm cultures. (**A**) Nunc 4-well plate culture of a 1 cm colony outgrowth (24 days) from a parthenogenetic bovine blastocyst (arrowheads indicate boundary of trophectoderm colony on the STO feeder cell layer), 6.4×. (**B**) Phase-contrast; light micrograph of primary IVP-blastocyst-derived bovine trophectoderm culture. 100×.

Experiment 2

Experiment 2 was undertaken to confirm the results of Experiment 1 and to examine IFN-τ production over time in tissue culture. As in Experiment 1, the success rate for trophectoderm colony-formation was similar for IVP and NT blastocysts, but was significantly lower for P blastocysts (Table 1). Method of blastocyst production, days in tissue culture and the Method × Day interaction were statistically significant (Table 3). For each day, IFN-τ activity in the CM of NT-derived trophectoderm primary colonies was significantly lower compared to IVP- and P-derived colonies, except at the day 23 assay point where the difference between P and NT was no longer significant at the $P = .05$ level (Table 4). Across all days in tissue culture, regardless of embryo production method, IFN-τ activity was highest at the earliest time point, 14 days in culture, and decreased significantly at each subsequent time point. Finally in Experiment 2, a comparison was made of the average size of the trophectoderm colonies by method of blastocyst production on the last day of culture, the 23rd day. Table 5 shows that, while the colony size means of IVP- and NT-derived colonies were not significantly different, the mean size of P-derived colonies was significantly reduced.

DISCUSSION

The results of this study show that IFN-τ protein secretion was often lower in primary trophectoderm

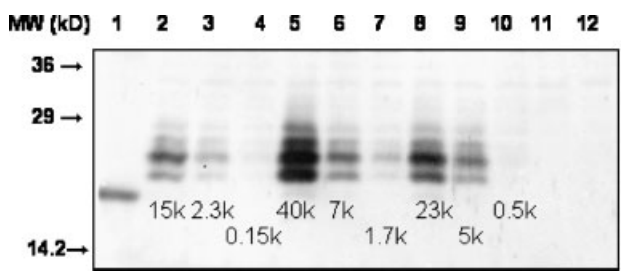

Fig. 2. Detection of differentially glycosylated IFN-τ isoforms (20–23 kDa) by reaction of anti-bovine-IFN-τ antiserum with a Western blot of NT-, parthenogenetic-, and IVP-derived primary trophectoderm CM. Signal density gives a semi-quantitative comparison of the bovine IFN-τ content of the CM samples. Samples were representative of high, medium, and low expression of anti-viral activity and the corresponding anti-viral titer ($k = 1000\times$) of each is displayed beneath the lane. Lane 1 = bovine recombinant IFN-τ (500 pg); lanes 2–4 are NT samples; lanes 5–7 are parthenogenetic samples; lanes 8–10 are IVP samples; lane 11 = STO feeder cell only CM; lane 12 = non-conditioned 10% DMEM.

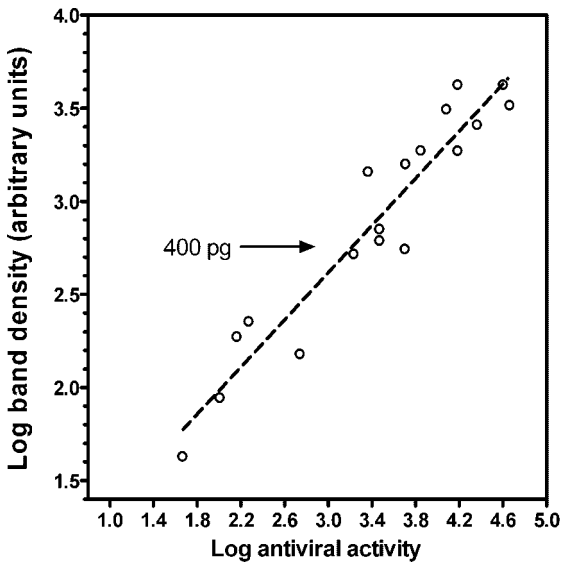

Fig. 3. Cartesian plot of densitometry of bovine IFN-τ detected by immunoblot vs. anti-viral activity titers in primary trophectoderm CM samples. Correlation coefficient = 0.91.

cultures of P- and NT-derived embryos in comparison to IVP-derived trophectoderm for some unknown reason. IFN-τ down-regulation in NT-derived trophectoderm cultures is probably not the result of genetic mutation or loss of genetic material as evidenced by the maintenance of microsatellite alleles in NT animals (Ashworth et al., 1998), the maintenance of previously established genetic defects in NT offspring (Blelloch et al., 2004), the generation of germ line competent embryonic stem (ES) cells from NT embryos (Wakayama et al., 2001), and the generation of cloned mice from tissues of various ages and types (Wakayama and Yanagimachi, 2001). On the contrary, examples of nuclear donor cells that would be expected to be mutated did not support the full-term development of NT embryos (Cui et al., 2003; Shi et al., 2003). Also, karyotype abnormalities were not found in early passage IVP and NT-derived trophectoderm cultures (Talbot et al., 2000a,b). What has been demonstrated is a dysfunction in the expression of a wide number of genes, both imprinted and nonimprinted genes, in NT embryos and NT placentas (Humpherys et al., 2002; Inoue et al., 2002). There is evidence that epigenetic changes, e.g., failure to reestablish normal DNA methylation patterns, result from NT, and it is these manifestations that may be the cause of what is described as insufficient nuclear reprogramming (Dean et al., 2001; Humpherys et al., 2001; Cezar et al., 2003; Santos et al., 2003).

Incomplete or aberrant DNA methylation of cis- or trans-acting gene expression control elements may be the cause of the lower IFN-τ secretion from the NT and P trophectoderm. This might arise from a lack of normally methylated DNA compliments in the parthenogenetic trophectoderm (Surani and Barton, 1983; Surani et al., 1986), or in the case of NT, result from ineffective reestablishment of genetic imprint status or methylation status (Humpherys et al., 2001; Cezar et al., 2003; Santos et al., 2003). For example, ovine IFN-τ gene expression has been shown to be affected by the methylation of specific 5′-upstream regions associated with the IFN-τ gene (Nojima et al., 2004). Downregulation of IFN-τ expression in the trophectoderm outgrowths could also result from effects on trans-acting elements that regulate the IFN-τ gene such as fos/jun complexes and Ets-2 (Xavier et al., 1997; Ezashi et al., 1998; Matsuda et al., 2004) or from other less defined complex genetic interactions such as the reported sex-linked expression of IFN-τ (Larson et al., 2001). Whatever the basis of the differential IFN-τ secretion is, some questions regarding the observation are: is it wholly or partially environmentally driven, is it an indicator of reprogramming deficiency, and is it an indicator of compromised embryo fitness or survival potential?

That in vitro models may be useful in tracking epigenetic errors resulting from NT is supported by the finding that in fibroblast cultures derived from uniparental fetuses (androgenotes or parthenogenotes), the parent-of-origin allele-specific expression profile of several maternally imprinted and several paternally imprinted genes were maintained over 30 cell generations in culture (Kharroubi et al., 2001). Presently, there is insufficient data to judge how mechanistically faithful the bovine trophectoderm cell lines are as in vitro models of the in vivo elongating bovine blastocyst. However, whether on feeders cells or not, the expression of IFN-τ from IVP-derived trophectoderm cell cultures and their demonstrated differentiation potential indicate that they probably are good models (Shimada et al., 2001; Nakano et al., 2002; Talbot et al., 2004b and unpublished observations). In any case, although the IFN-τ expression in the primary trophectoderm cultures might be different from what would occur in vivo, their assay under identical egg culture and tissue culture conditions enables the comparison of their genetic and epigenetic status relative to one another. Differences in IFN-τ expression resulting from epigenetic changes that result from NT reprogramming failures might therefore be observable against the background of genetic differences resulting from cell culture selection pressures and differences in parental origin. The latter being of concern because within the IVP group sperm from three bulls was used in Experiment 1, and for both IVP and P, the eggs were obtained from slaughterhouses ovaries. Similarly, within the NT group, three cell lines were used as nuclear donors in Experiment 1 (albeit that they were genetically from a single source, i.e., clone of a clone origin for BFF 101 and BFF 13). Despite these sources of variation, the down-regulation of IFN-τ in the NT outgrowths suggests failures in nuclear reprogramming as the cause, and this could be potentially useful in defining aspects of NT methodology that produce undesirable or unnatural effects in bovine embryos.

A previous study of intact bovine blastocysts found a similar reduction in the expression of IFN-τ from NT embryos as presented here, although other studies

involving IVP and P bovine blastocysts do not agree in all aspects with our findings. Stojkovic et al. (1999), found in a "long-term" individual blastocyst per drop culture system that the IFN-τ expressed by NT bovine embryos was reduced compared to the amounts produced by in vivo- or IVP-derived embryos. Also, besides producing higher amounts of IFN-τ than NT embryos, the in vivo- and IVP-derived bovine embryos produced similar amounts of IFN-τ (Stojkovic et al., 1999). Perhaps in contrast to our findings, however, are those of Larson et al. (2001) that showed female blastocysts produced approximately twice as much IFN-τ as male blastocysts. In the present study, the NT trophectoderm outgrowths were all derived from female blastocysts since the nuclear donor cell lines used (BFF 10, 13 and 101) were female. Likewise, the P-derived trophectoderm cultures were maternal in genome complement. IVP trophectoderm outgrowths were presumably approximately 50/50 female and male. Therefore, a significant sex bias existed in the experimental design, and one that might have been expected to favor greater production of IFN-τ by the NT and P outgrowths. Since the opposite result was found, either the assay of intact blastocysts presented in Larson et al. (2001) is not comparable to the primary cultured trophectoderm, or they are comparable and epigenetic differences present in NT and P embryos were contributing to the IFN-τ down regulation observed.

Antiviral activity from P-derived outgrowths was intermediate compared to IVP (highest) and NT (lowest) in the present study. It might be expected that parthenogenetic embryos would yield trophectoderm outgrowths with less IFN-τ production since the embryos are epigenetically deficient, cannot produce viable embryos, and, at least in the mouse, have poor development of extraembryonic tissue (Surani and Barton, 1983). Despite these expectations, and directly opposite to our results, it was recently shown that parthenogenetic bovine blastocysts and female IVP blastocysts produced more IFN-τ than male IVP blastocysts (Kubisch et al., 2003). In the same study, tissue culture blastocyst outgrowths grown on Matrigel coated plates did not show a difference in IFN-τ production between IVP and parthenogenic blastocysts, again in contrast to our results (Kubisch et al., 2003). In a similar study (Kubish et al., 2001), IVP blastocysts produced different levels of IFN-τ depending on day of blastocyst formation (i.e., seventh or nineth day) in the first 48–96 h of culture and also showed significant differences depending on the egg source, i.e., "ovary batch". On subsequent tissue culture of the blastocysts on Matrigel coated plates, the blastocoel cavity formation differences (for 7-day compared to 9-day) disappeared by the 6th day of culture, and by the 12th day of culture were also no longer significantly different by ovary batch (Kubisch et al., 2001). Similarly, analysis of our data based on length of time in egg culture (not the point of blastocoel formation) showed a statistically significant reduction in IFN-τ expression from cultures derived from blastocysts kept in egg culture until the 10th day, i.e., approximately 2 days after blastocoel formation (data not shown). Together these results appear to indicate the potential for changes in IFN-τ expression depending on the "health" of the blastocyst (egg culture effect), and depending on the adequacy of the tissue culture environment for cell growth and function (cell culture effect). The STO feeder cell environment utilized for these studies may provide a better model than extended egg culture (Stojkovic et al., 1999; Kubisch et al., 2001) or Matrigel culture (Kubisch et al., 2003) because the STO feeder cells have been shown to stimulate rapid bovine trophectoderm cell growth and enable the establishment of bovine trophectoderm cell lines (Talbot et al., 2000b, 2004b). This may in part explain the differences between our findings concerning the differential production of IFN-τ from IVP- versus. P-derived trophectoderm and those reported by Kubisch et al. 2003.

In the present study the incidence of colony-formation and colony size did not differ between IVP-derived and NT-derived blastocysts whereas significantly fewer P-derived blastocysts were able to form colonies and the colonies produced were on average smaller. This is in contrast to the results of Kubisch et al., 2003 where P and IVP blastocyst outgrowths occurred at a similar efficiency. Perhaps Matrigel is better able to support the initial attachment and spreading of trophectoderm cells compared to STO feeder cells since Kubisch and co-workers further reported that the parthenogenetic outgrowths were initially larger than IVP outgrowths, but that this difference disappeared by the 14th day of tissue culture (Kubisch et al., 2003). Alternatively, or in addition to, the parthenogenic blastocysts produced by Kubisch and co-worker's egg culture system may have been of better quality compared to those produced in our serum-free and feeder-independent egg culture system. In any case, the main significance of the colony-formation and colony size comparisons is that IVP and NT blastocysts were alike in these respects, and, therefore, does not provide an explanation for the low levels of IFN-τ produced by the NT trophectoderm outgrowths.

Experiment 2 was undertaken to confirm the results of Experiment 1 since there were many uncontrolled variables in Exp. 1, and, also, some pre-selection of NT embryos occurred in Experiment 1 because of the necessity to share some embryos with the laboratory's concurrent in vivo cloning initiative. While Experiment 2 did confirm the findings of Experiment 1, i.e., that NT blastocyst outgrowths were comparatively deficient in IFN-τ secretion, it was also of interest to ascertain if the assay time could be shortened and at what time in the primary trophectoderm culture maximal IFN-τ secretion occurred. It had been assumed prior to the start of Experiment 1 that the IFN-τ expression would positively correlate with colony size, but as shown by Experiment 2, colony size, or time in tissue culture, was negatively correlated with IFN-τ secretion. This may have resulted from increasing amounts of cell death from apoptosis and necrosis within the primary

trophectoderm colonies as the colonies grew older and larger. However, this is only conjecture based on microscopic inspections of the cultures as they grew over time. In any case, Experiment 2 demonstrated that the IFN-τ secretion could be detected by at least as early as 14 days after initiation of the primary culture, and, in fact, the highest levels of IFN-τ were found at this earliest time point. If a smaller tissue-culture-well format was used, it seems likely that the assay could be shortened even further since the ratio of cells to tissue culture fluid would be reduced.

The reduced secretion of IFN-τ from NT-derived outgrowths may indicate one factor that contributes to the failure of NT embryos to establish pregnancies that carry to term. In relation to this, an effect of systemic or intrauterine administration of IFN-τ to improve pregnancy and birth rates in cattle and sheep has not been reported. However, one study in red deer involving asynchronous embryo transfers did show a dramatically beneficial effect on birth rates from the systemic injection of IFN-τ (Demmers et al., 2000). Also, a correlation between the IFN-τ production by IVP or NT embryos and subsequent pregnancy success after embryo transfer remains to be established. Indeed, a recent pertinent report showed that there was a negative correlation between IFN-τ production in IVP blastocyst-stage bovine embryos and pregnancy rate after embryo transfer (Kubisch et al., 2004). Thus, although the production of IFN-τ at the appropriate time and in the correct amounts is thought to be critical for maternal recognition of pregnancy (Roberts et al., 1990) and placentation (Klein et al., 2006), it remains to be demonstrated that IFN-τ expression at the blastocyst stage can be a predictor of placental fitness or pregnancy success. Likewise, since the quality and relevance of trophectoderm cultures as in vitro models of the elongation-phase of bovine blastocyst is speculative at this point, it is also not presently possible to say that the low IFN-τ levels found in the NT outgrowths are faithful predictors of pregnancy failure; or potential pregnancy success in those NT embryos that produce outgrowths with IVP-like IFN-τ secretion levels.

However, and in conclusion, the assay of IFN-τ from blastocyst outgrowths may serve as a useful tool for identifying methods that generate better NT embryos in terms of normal gene expression. Further, NT pregnancy losses often occur shortly after placentation, and NT placental abnormalities are often found at all stages of gestation (Hill et al., 2000; De Sousa et al., 2001; Tanaka et al., 2001; Humpherys et al., 2002; Lee et al., 2004; Powell et al., 2004). This, combined with recent evidence that placental and trophectoderm DNA methylation and gene expression is abnormal in NT embryos (Kang et al., 2002; Singh et al., 2004), would suggests that analysis of trophectoderm tissue gene expression, particularly IFN-τ expression (Stewart et al., 2001; Klein et al., 2006), may logically yield mechanistic information on NT pregnancy failure and NT reprogramming.

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